STUDIES ON THE PURIFICATION AND CHARACTERIZATION OF DIPEPTIDYLAMINOPEPTIDASE. IV.

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16. Abstract

In the microsomal of pig kidneys, aside from particle-bound aminopeptidase (EC 3.4.1.2), a dipeptidyl aminopeptidase is observed which, upon solubilization, fractionated ammonium sulfate precipitation and column chromatography, can be separated from the aminopeptidase, and isolated purely by disc electrophoresis. The present studies have led to the assumption that dipeptidyl aminopeptidase IV possesses two different specifities for substrates with the sequence L-AS-L-Ala-R, and for substrates with the sequence L-AS-L-Pro-R. The enzymatic hydrolysis of the two substrate sequences differs by the position of pH optima and the inhibition by DFP. Whereas cations have a relatively weak influence on the enzymatic activity of dipeptidyl aminopeptidase IV, the influence of anions proved to be significant.

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STUDIES ON THE PURIFICATION AND CHARACTERIZATION OF DIPEPTIDYL AMINOPEPTIDASE IV.

A. Barth, H. Schulz and K. Neubert

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Introduction

Dipeptidyl aminopeptidase IV, first demonstrated in hog kidney microsomes by Hopsu-Havu and Glenner [1] in 1965, is one of the group of proteolytic enzymes characterized by particular specificity for proline-containing peptides [2]. According to the studies by Hopsu-Havu [3] and other authors, dipeptidylaminopeptidase IV optimally hydrolyzes both L-Ala-L-Ala-β-Naphthylamide and Gly-L-Pro-β-naphthylamide. In its physical chemical characteristics, it appears to match the dipeptidylaminopeptidases with the same action which have been isolated from other types of organs, such as liver [4], rat skin [5-7], human skin [7], human serum [8] and parotid gland [9, 10].

Only a limited number of dipeptidylarylamides were used /158
as substrates in all the previous studies to characterize the action and specificity. This lack of range in variation of the peptide sequence of the arylamides did not allow final statements about any "in vitro specificity" of the dipeptidylaminopeptidase IV. It is of interest, then, to undertake characterization of the highly purified enzyme using a larger number of dipeptidylarylamides with different amino acid esters.

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* Numbers in the margin indicate pagination in the original foreign text.

Materials and Methods

Twenty-four dipeptidyl-4-(phenylazo)-phenylamides [11, 12] were used for the substrate specificity studies. They were synthesized in steps by the mixed anhydride method and were pure according to thin layer chromatography. The substrates L-alanyl-L-alanyl-L-alanine-PAP-amide and L-alanyl-L-alanyl-L-alanyl-L-alanine-PAP-amide were obtained by the same method. [PAP = 4-(phenylazo)-phenyl residue]. They were used as the hydrochlorides in studying the step by step mechanism.

The following reagents were used for chemical modification of the dipeptidylaminopeptidase IV: diisopropylfluorophosphate (DFP) from the Ferrak Company, Berlin; p-chloromercuribenzoate (pCMB) from the company EGA-Chemie and Keppler and Reif, KG; chloroacetamide from the Laborchemie Peoples Factory, Apolda; 2-hydroxy-5-nitrobenzyl bromide from the Ferrak Company, Berlin; 1,4-dibromo-2-phenylacetoin, synthesized according to [17]; L-1-tosylamido-2-phenylethylchloromethylketone, synthesized according to [18]; and o-phenanthroline from the Chemapol Company, Prague.

The cations tested as effectors for the dipeptidylaminopeptidase were used in as the acetates, and the anions as their sodium or potassium salts, in the commercial p. A. quality.

The enzymatic activity was determined in the discontinuous or continuous methods according to Barth et al. [13, 14] and Senkpiel [15], respectively. In the former case, the 4-(phenylazo)-phenylamine (PAP) liberated enzymatically is determined spectrophotometrically at 500 nm after acidification of the reaction mixture with 40% trichloroacetic acid. In the second case, the decrease in substrate is followed at 325 nm during

the enzymatic process without interrupting the reaction.

The enzyme activity under pH-stat conditions was done by incubation in an Autotitrator-Titrigraph TTT 1c/SBR 2c from the Radiometer Company (Copenhagen), combined with spectrophotometric evaluation at 500 nm with a VSU 1 from the Carl Zeiss Peoples Factory (Jena).

The hydrolysis products from the substrates, L-alanyl-L-alanyl-L-alanine-PAP-amide and L-alanyl-L-alanyl-L-alanyl-L-alanine-PAP-amide were separated by paper electrophoresis at 400 volts, using Precipitate Paper F-14 (moderately fast) and 7% acetic acid as the bath liquid. After concluding the separation, the electropherogram was dried for 20 minutes at 100°C and developed with ninhydrin-cadmium acetate reagent [16]. The substrate and the fission product, 4-(phenylazo)-phenylamine can be detected well from the beginning due to their own color, so that a good differential demonstration is possible if dipeptide fission products are superimposed on these materials.

Enzyme Preparation (Table 1):

1. Obtaining the microsome fraction and enzyme solubilization

Three kilograms of fresh hog kidneys were freed from the inner marrow, cut into cubes, and ground twice in a mincing machine. The paste was then suspended in 0.25 M sucrose solution (about 1:6 v:v) and homogenized with a high-speed Ultra-Turrax from the Janke and Kunkel company. In order to isolate the microsome fraction, the homogenate was then centrifuged for 60 minutes at 3,000 g and then repeatedly at 17,000 g. The subsequent ultracentrifugation at 150,000 g for 120 minutes led to sedimentation of the microsomes. / 159

Table 1. CHARACTERIZATION OF PURIFICATION STEPS IN ISOLATION OF DIPEPTIDYLAMINOPEPTIDASE IV.

Purification step	Total Volume [ml]	Total Protein [mg]	Total Activity [U]	Specific Activity [U/mg]	Purification Factor (1)
Kidney homogenizate	2700	12826	–	–	–
Solubilized protein fraction after autolysis	340	5430	5,43	0,001	1
Protein precipitation on 85% $(NH_4)_2SO_4$ saturation and 48 hours dialysis	23	1340	25,46	0,019	99
Sepharose 4B separation	2,7	208	20,17	0,097	97
Chromatography on Sephadex DEAE A-50 ion exchanger	3,4	38	6,84	0,180	180
Re-chromatography on Sepharose 6B	2,6	28,6	6,57	0,230	230

(1) Total and specific activities were determined with the substrate L-Ala-L-Ala-PAP-amide. One unit is defined as 1 µmol PAP amine liberated per minute at 30°C and a substrate concentration of $2 \cdot 10^{-4}$ M (substrate saturation)

To obtain the crude enzyme extract, the microsomes were suspended in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 and autolyzed for 20 hours at 37°C.

2. Fractional ammonium sulfate precipitation and dialysis

After autolysis, the clear crude enzyme extract was subjected to fractional protein precipitation. Sedimentation was done stepwise at 0 - 40% and then at 40 - 85% ammonium sulfate saturation at intervals of 24 hours. The protein

precipitate at 85% saturation with ammonium sulfate was dialyzed for 48 hours versus 1 mM phosphate buffer, pH 7.7 (the dialysis buffer was changed after 24 hours) and used for gel filtration after concentration.

3. Gel filtration on Sepharose 4B

Gel filtration was done on a 3 x 70 cm column after the crude enzyme was obtained as described above. Elution medium: phosphate buffer, 0.02 M, pH 7.3, flow rate 20 ml/hour.

4. Ion exchange chromatography on Sephadex DEAE A-50

After gel filtration, the enzymatically active fraction was subjected to chromatography on an ion exchanger. A column 2.5 x 25 cm was used. The enzyme was desorbed with a linear NaCl gradient (0.07 - 0.7 M) in 0.02 M phosphate buffer, pH 7.3. Flow rate: 15 ml/hour.

5. Rechromatography on Sepharose 6B / 160

Rechromatography was done with Sepharose 6B on a 2.5 x 45 cm column to obtain highly purified dipeptidylaminopeptidase. The elution medium was 0.02 M phosphate buffer at pH 7.3.

Determination of the molecular weight

The molecular weight of the dipeptidylaminopeptidase IV was determined on a column of Sephadex G-200 (1.5 x 90 cm) from Pharmacia, Uppsala. The calibration proteins used were: apoferritin, MW 480,000 (Serva, Heidelberg); particle-bound hog kidney aminopeptidase, MW 280,000 (prepared according to Pfleiderer [37]; human gamma globulin, MW 160,000 (Serva, Heidelberg). Dextran Blue was used to determine the exclusion volume (Serva, Heidelberg).

Determination of the isoelectric point

The isoelectric point was determined at various pH values in 1% agarose gel. Buffer system: 0.02 M Tris-maleic acid, pH 5 to 8, 60 V, 60 minutes. The reference protein was human serum albumin.

Analytical polyacrylamide gel electrophoresis

This process was performed according to specifications by Maurer [21] in 7% polyacrylamide gel and discontinuous Tris-glycine buffer (5 mM, pH 8.4) for 90 minutes at 300 V and 2 mA in electrophoresis tubes.

Immunoelectrophoresis

In order to obtain the antiserum, rabbits (New Zealand) were injected with the enzymatically active protein fraction obtained after the Sepharose 4B gel filtration.

The antigen-antibody reaction was studied in analogy with the immunoelectrophoresis method according to Schneidegger [23].

Protein determination

Protein was determined by the method of Lowry et al. [19], as modified by Gläser and Kleine [20].

Results and Discussion

It is known from the studies of Hopsu-Havu and Sarimo [4] that the highest enrichment of dipeptidylaminopeptidase IV is gained through autolysis. In these studies, this method was used in combination with addition of 0.1% Triton X-100, followed by fractional ammonium sulfate precipitation. In the remaining

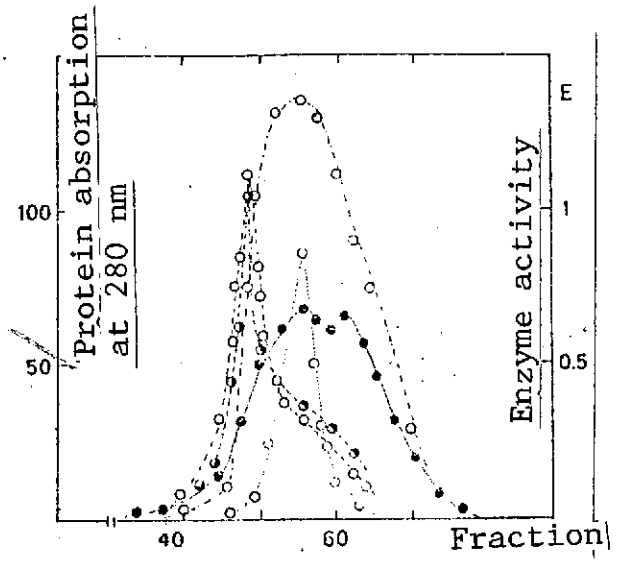

Figure 1. Gel filtration on Sepharose 4B.
●——● = protein spectrum; ○-·-○ = aminopeptidase activity (substrate: L-Ala-PAP-amide); ○-·-○ = esterase activity (substrate: α-naphthyl acetate); ○···○ = phosphatase activity (substrate: p-nitrophenyl phosphate); ○------○ = dipeptidylaminopeptidase activity (substrate: L-Ala-L-Ala-PAP-amide).

enzyme purification operations, separation of the desired dipeptidylaminopeptidase from the accompanying particle-bound aminopeptidase (EC 3.4.1.2) proved problematical. Here, the coupling of gel filtration on Sepharose 4B with subsequent ion exchange chromatography on Sephadex DEAE A-50 turned out to be an essential step for complete and clean separation of the dipeptidylaminopeptidase from particle-bound aminopeptidase.

As is shown in Figure 1, both enzyme activities (dipeptidylaminopeptidase and aminopeptidase) are still apparent in the same peak after the first gel filtration. The only intent of this step was to separate mose of the foreign proteins, thus preparing for the following separation of the two enzymes. The

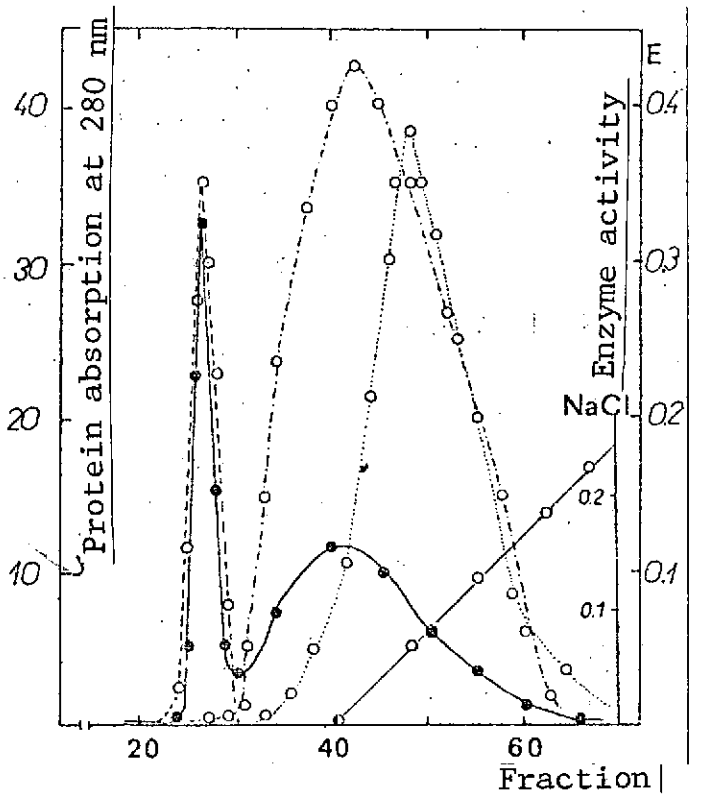

Figure 2. Ion exchange chromatography on Sephadex DEAE A-50. c—c = protein spectrum; o--o = dipeptidyl-aminopeptidase activity; o·····o = aminopeptidase activity; o-·-·-o = esterase activity; o——o = NaCl gradient (substrates as in Figure 1).

dipeptidylaminopeptidase and the aminopeptidase separate from each other only during subsequent ion exchange chromatography on Sephadex DEAE A-50 (Figure 2). At the same time, an unspecific esterase activity is completely eliminated. The enzyme is highly purified by rechromatography on Sepharose 6B. Disc electrophoresis studies done in parallel (Figure 3) show that all the separable accompanying proteins are eliminated in this way. The dipeptidylaminopeptidase migrates homogeneously in one band. No extraneous activities, which would indicate contamination by esterases or aminopeptidases, can be shown in the disc electropherogram.

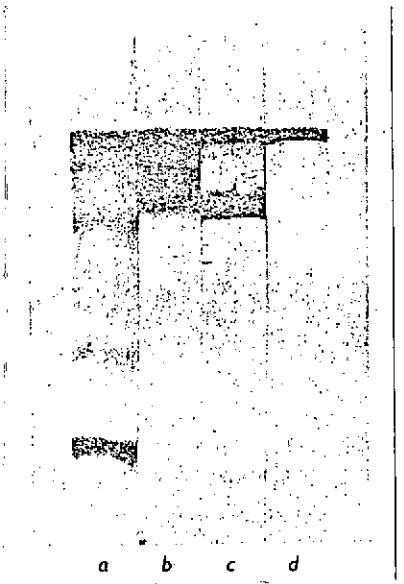

Figure 3

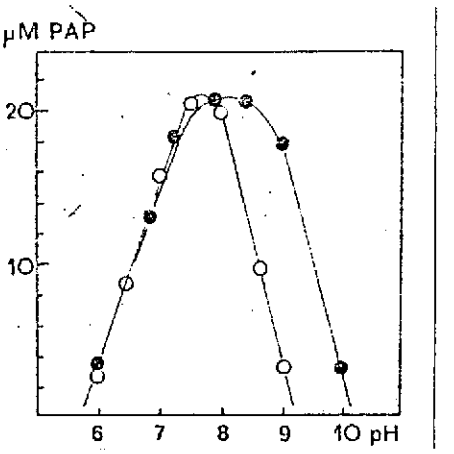

Figure 4

Figure 3. Discontinuous polyacrylamide gel electrophoresis. a = autolysis fraction; b = sepharose 4B fraction; c = Sephadex DEAE A-50 fraction (electrophoresis of the peak with aminopeptidase activity); d = Sephadex DEAE A-50 fraction (electrophoresis of the dipeptidylaminopeptidase peak).

Figure 4. Plot of the pH effect on the hydrolysis of L-Ala-L-Ala-PAP-amide (o——o) and Gly-L-Pro-PAP-amide (●——o) . Substrate concentration: $1 \cdot 10^{-4}$ M, Theorell-Stenhagen buffer, pH 7.7, 30°C.

Experiments to Characterize the Dipeptidylaminopeptidase IV.

1. pH dependence

The dependence of the dipeptidylaminopeptidase catalytic activity on the pH value of the reaction medium was studied over the range from pH 6.0 to 10.0 using Theorell-Stenhagen buffer. The result is shown in Figure 4. It appears that the optimum pH is 7.7 for hydrolysis of the substrate L-alanyl-L-alanine-4-(phenylazo)phenylamide (pK = 6.70),

and that the optimum pH is 8.1 for hydrolysis of the substrate glycyl-L-proline-4-(phenylazo)-phenylamide (pK = 6.45).

2. Temperature dependence / 162

The effect of the temperature on the enzymatic hydrolysis of the substrate L-alanyl-L-alanine-4-(phenylazo)-phenylamide was determined in the range from 15°C to 70°C. As appears from Figure 5, the highly purified dipeptidylaminopeptidase IV has a sharp temperature optimum at 52°C, for action on L-Ala-L-Ala-PAP-amide. Higher temperatures cause rapid inactivation of the enzyme.

3. Influence of effectors

For further characterization of the dipeptidylaminopeptidase IV, the influence of a series of anions, cations, and chemical modification reagents on the enzymatic activity was studied. The substrate was L-alanyl-L-alanine-PAP-amide. The results of these studies are shown in Table 2.

Of the anions, those with high charge density, such as phosphate and citrate, prove to be pronounced inhibitors. Cations show little to no effect. Only at higher concentrations of, for instance, $2 \cdot 10^{-3}$ M is there inhibition even from $Cu^{2+} < Mo^{6+} < Fe^{3+} < Hg^{2+}$. / 164

It is interesting to note that, in contrast to the observations of Hopsu-Havu et al. [3, 4], the enzymatic activity of the hog kidney dipeptidylaminopeptidase IV is severely reduced by DFP. As appears from Figure 6a, the catalytic action, referred to hydrolysis of the substrate L-alanyl-L-alanine-PAP-amide, is severely inhibited in an inhibitor concentration range of $1 \cdot 10^{-6}$ to $1 \cdot 10^{-5}$ M. This inhibiting

Table 2. INFLUENCE OF EFFECTORS ON THE ACTIVITY OF DIPEPTIDYL-AMINOPEPTIDASE IV ON L-ALA-L-ALA-PAP-AMIDE AS THE SUBSTRATE. (DATA ON EFFECTOR ACTION IN %, REFERRED TO THE ENZYMIC ACTIVITY WITHOUT EFFECTOR = 100%).

Effector	Concentration [M]			Effector	Concentration [M]	Effector	Concentration [M]
	$2 \cdot 10^{-4}$	$2 \cdot 10^{-5}$	$2 \cdot 10^{-6}$		$2.5 \cdot 10^{-2}$		$1 \cdot 10^{-3}$
				Phosphate	46,5	DFP	0
				Citrate	39,0	PMSF	56
Mg^{2+}	100	95	95	Borate	74,0	pCMB(1)	86
Ca^{2+}	100	96	93	Acetate	80	Chloro-acetamide	101
Cu^{2+}	99	97	24	Sulfate	49		
Cd^{2+}	100	99	61	AMP	57,5	HNB	81
Hg^{2+}	67	8	1	Fluoride	61,5	HNC	87
Mo^{6+}	96	76	9	Chloride	65	DPA	108
Mn^{2+}	99	98	83	Bromide	67	TPCK	115
Fe^{3+}	99	91	7	Iodide	71,5	EDTA	85
Ni^{2+}	100	100	87	Thio-cyanate	71,5	o-phen.	71
Co^{2+}	100	100	96				

DFP: diisopropylfluorophosphate
PMSF:
pCMB: p-chloromercuribenzoate
HNB: 2-hydroxy-5-nitrobenzyl bromide
HNC:
DPA: 1,4-dibromo-2-phenylacetoin
TPCK: L-1-tosylamido-2-phenylethylchloromethyl ketone
EDTA: ethylenediaminetetraacetic acid
o-phen: o-phenanthroline
(1) Used at a concentration of $1 \cdot 10^{-4}$ M.

effect of DFP depends on the preincubation time (Figure 6b). The expression of maximum inhibition is then attained at a preincubation time of about 120 minutes. The half-peak time / 165
for the inhibition is about 30 minutes. On the basis of this experiment, the kinetic studies to characterize the DFP effect used 120 minutes of preincubation, if not otherwise noted,

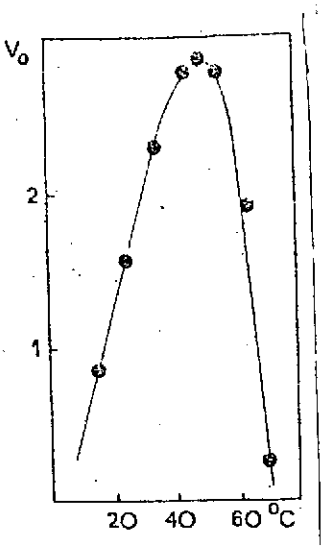

Figure 5. Graph of the effect of temperature on the rate of hydrolysis of L-Ala-L-Ala-PAP-amide. Substrate concentration: $1 \cdot 10^{-4}$ M, Theorell-Stenhagen buffer, pH 7.7, preincubation 20 minutes, 30°C.

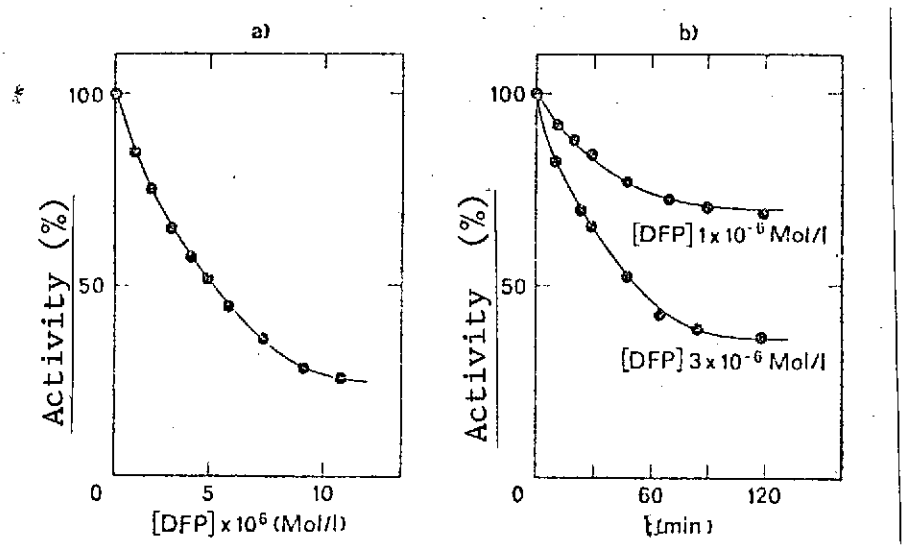

Figure 6. a) Relative activity (%) of dipeptidylaminopeptidase IV as a function of the DFP concentration. Substrate: L-Ala-L-Ala-PAP-amide ($1 \cdot 10^{-4}$ M), Theorell-Stenhagen buffer, pH 7.7, temperature 30°C, preincubation time 30 minutes.
b) Change in enzymic activity of dipeptidylaminopeptidase IV as a function of the DFP preincubation time. Buffer and temperature as in Figure 6a.

after which the reaction with substrate is started.

Evaluation of the DFP-inhibited hydrolysis of L-Alanyl-L-Alanine-PAP-amide by dipeptidylaminopeptidase IV with the Lineweaver-Burk method showed a noncompetitive type of inhibition. The Hill coefficient was determined as n = 1.5. All the other modification reagents tested, including the complexing agents EDTA and o-phenanthroline, showed no activity.

As Figure 7 shows, the inhibiting effect of DFP differs, depending on the type of substrate used. A pI_{50} value of 5.30 was found for hydrolysis of L-Alanyl-L-Alanine-PAP-amide. In contrast, the pI_{50} value for inhibition of dipeptidylaminopeptidase by DFP is 4.60 for hydrolysis of the substrate glycyl-L-proline-PAP-amide.

The difference between these experimental results for inhibition of dipeptidylaminopeptidase IV by DFP and the literature values [3, 4] could be due to the fact that the Hopsu-Havu group tested the inhibitory effect of DFP on dipeptidylaminopeptidase IV from rat liver. Studies with the corresponding enzyme from hog kidney were limited only to study of the effect of the phosphoric acid ester Mintacol (E 600). It is quite possible that there is species-specific inhibition by DFP.

The effect of urea on dipeptidylaminopeptidase IV has not previously been tested. In the present studies, the highly purified enzyme proved extremely stable to 8 M urea. No significant loss in activity occurred over a period of 4 hours. Only after increasing the urea concentration to 12 M in the incubation solution was the dipeptidylaminopeptidase inactivated after 4 hours. As appears from disc electrophoretic studies, this inactivation is linked with protein aggregation (Figure 8).

The native enzyme, under the action of 12 M urea, gives an associate which no longer penetrates into 7% polyacrylamide gel. An approximate molecular weight of 480,000 was estimated using gel filtration on Sephadex G-200. Apparently the enzymatically active protein dimerizes. It is not yet clear whether this dimerization process is a simple "doubling" or whether it is an aggregation of intermediate subunits. Such behavior is surprising and, to our knowledge, has not been observed with other aminopeptidases. Further studies are planned to clear up this problem.

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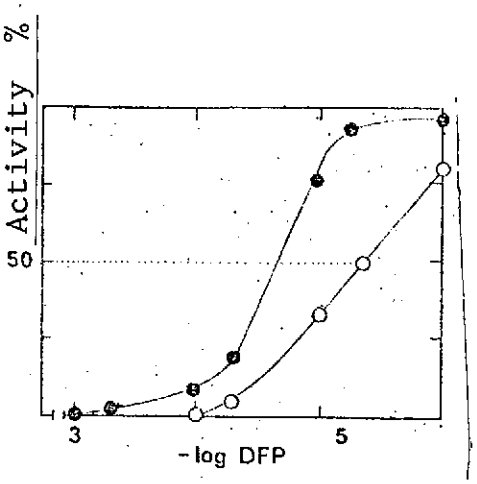

Figure 7

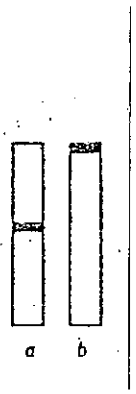

Figure 8

Figure 7. Effect of the DFP concentration on the enzymatic hydrolysis of Gly-L-Pro-PAP-amide (o—o) and L-Ala-L-Ala-PAP-amide (o—o). Substrate concentration: $1 \cdot 10^{-4}$ M. DFP concentration in the range of $1 \cdot 10^{-7}$ to $1 \cdot 10^{-3}$ M. Theorell-Stenhagen buffer, pH 7.7, 30°C, preincubation time 30 min.

Figure 8. Pherograms of dipeptidylaminopeptidase in 7% polyacrylamide gel. a = native enzyme; b = after 4 hours treatment with 12 M urea.

Table 3. SPECIFICITY OF DIPEPTIDYLAMINOPEPTIDASE IV FOR DIDPEPTIDYL-4-(PHENYLAZO)-PHENYLAMIDE SUBSTRATES. (THE RELATIVE ACTIVITY IS REFERRED TO THE HYDROLYSIS OF L-ALA-L-ALA-PAP-AMIDE = 100%).

Substrate	Activity		Substrate	Activity	
	Specific (U/mg)	Relative (%)		Specific (U/mg)	Relative (%)
L-Ala-L-Ala-PAP-amide	0,14	100	D-Ala-L-Ala-PAP-amide	0	0
Gly-L-Ala-PAP-amide	0,026	20	L-Ala-D-Ala-PAP-amide	0	0
L-Lys-L-Ala-PAP-amide	0,102	70	D-Ala-Gly-PAP-amide	0	0
L-Pro-L-Ala-PAP-amide	0,18	127	L-Ala-Gly-PAP-amide	0	0
L-Met-L-Ala-PAP-amide	0,22	152	L-Ala-L-Leu-PAP-amide	0	0
L-Leu-L-Ala-PAP-amide	0,28	200	Gly-Gly-PAP-amide	0	0
L-Phe-L-Ala-PAP-amide	0,67	470	Gly-L-Phe-PAP-amide	0	0
			L-Leu-L-Leu-PAP-amide	0	0
L-Leu-L-Pro-PAP-amide	0,69	490	L-Leu-Gly-PAP-amide	0	0
Gly-L-Pro-PAP-amide	0,73	520	L-Met-L-Met-PAP-amide	0	0
L-Phe-L-Pro-PAP-amide	0,92	660	L-Phe-L-Phe-PAP-amide	0	0
L-Pro-L-Pro-PAP-amide	0,98	700	L-Pro-Gly-PAP-amide	0	0
L-Ala-L-Pro-PAP-amide	1,08	770	Boc-L-Leu-L-Ala-PAP-amide	0	0

4. Substrate specificity

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Twenty-four dipeptidyl-PAP-amides with different amino acid sequences were tested to determine the "substrate specificity" of the dipeptidylaminopeptidase. In the evaluation of the substrate specificities of dipeptidylaminopeptidase I [36], II [34], and III [30], which have already been described by other authors, dipeptidyl-PAP-amides with increasing hydrophobicity of the amino acid side chains were tested preferentially.

As can be seen from Table 3, only substrates with the sequences L-amino acid-L-Ala-PAP-amide and L-amino acid-L-Pro-PAP-amide were hydrolyzed by the dipeptidylaminopeptidase IV. Amino acids of the L-configuration are of equal importance to the presence of an unprotected N-terminal amino group for the

hydrolysis mechanism. D-amino acid-PAP-amides and dipeptide esters such as L-Ala-L-AlaOCH_3 are not converted by the dipeptidylaminopeptidase.

By comparing the substrates listed in Table 3 and their specific activities, we can see that it is only the nature and the manner of arrangement of the amino acids in the dipeptidyl substrates which decides their hydrolysis.

Essentially, we must ascribe the expression of specificity to the N-terminal amino acids, while the following amino acid in position 2 causes the selectivity and decides whether hydrolysis occurs or not.

Thus, the dipeptidylaminopeptidase IV has two different substrate specificities, one for substrates with the sequence L-amino acid-L-Ala-R and the other for substrates with the sequence L-amino acid-L-Pro-R. Figure 9 shows that in the substrate series L-amino acid-L-Ala-PAP-amide (below) the specific activity increases continuously with increasing hydrophobicity of the N-terminal amino acid side chain. In the substrate series L-amino acid-L-Pro-PAP-amide (above), there is no such dependence. In this case, hydrophobic interactions are probably coupled with steric factors. In this respect it is interesting that in the alanine series, N-terminal leucine contributes to an increase in the activity, while in the proline series it reduces the specific activity, most probably through steric hindrance (see Figure 10). The effects observed on the hydrolysis of the substrates L-Pro-L-Pro-PAP-amide and L-Pro-L-Ala-PAP-amide are also worth mention. In the lower series of Figure 9, we should expect a specific activity of about 0.6 U/mg for the enzymatic hydrolysis of L-Pro-L-Ala-PAP-amide, corresponding to the hydrophobic character of the proline residue. But in fact, only 0.18 U/mg is measured.

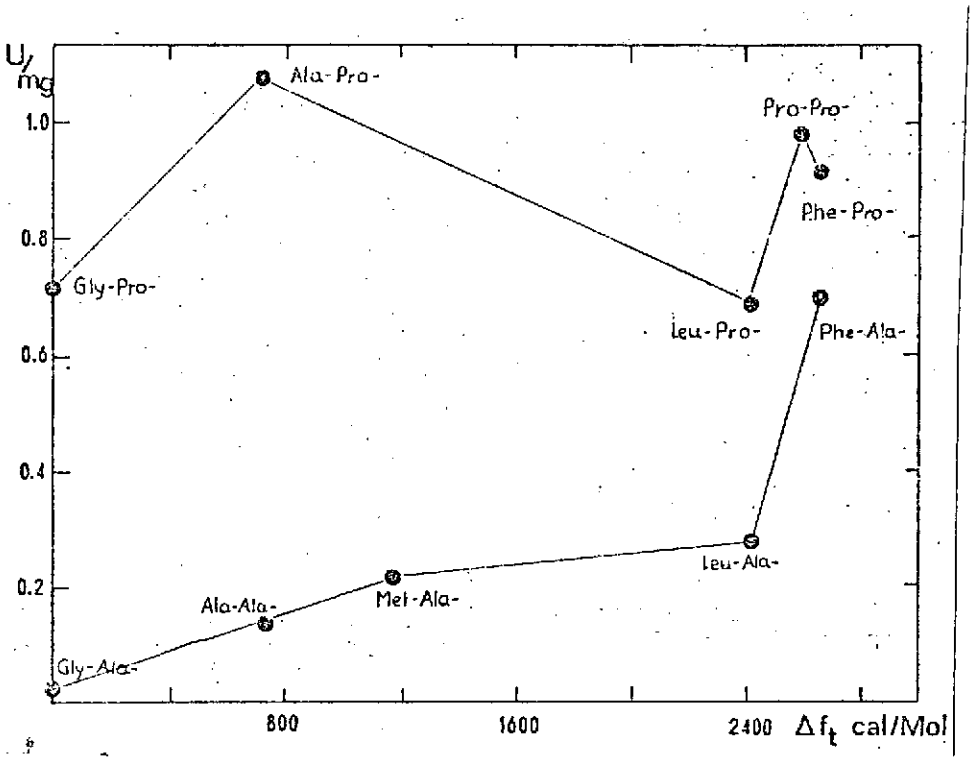

Figure 9. Representation of the specific activity (U/mg) for hydrolysis of substrates of the types L-amino acid-L-Ala-PAP-amide and L-amino acid-L-Pro-PAP-amide as functions of the hydrophobic character of the side chain of the N-terminal amino acid (according to Nozaki and Tanford [33], the change in the free energy, Δf_t (cal/mol) of the amino acid side chain in the system ethanol/water is a measure of the hydrophobicity).

The presence of a primary N-terminal amino group is also important for substrates of this type. But in the upper series of Figure 9, L-Pro-L-Pro-PAP-amide is one of the "most active" substrates. Here the condition of the presence of a primary N-terminal amino group does not seem so important. It must be noted here that double substrate specificities have already / 168 been described for the dipeptidylaminopeptidases II and III [30, 34]. From the structural models of some substrates (Figure 10), on consideration of the most favorable angular

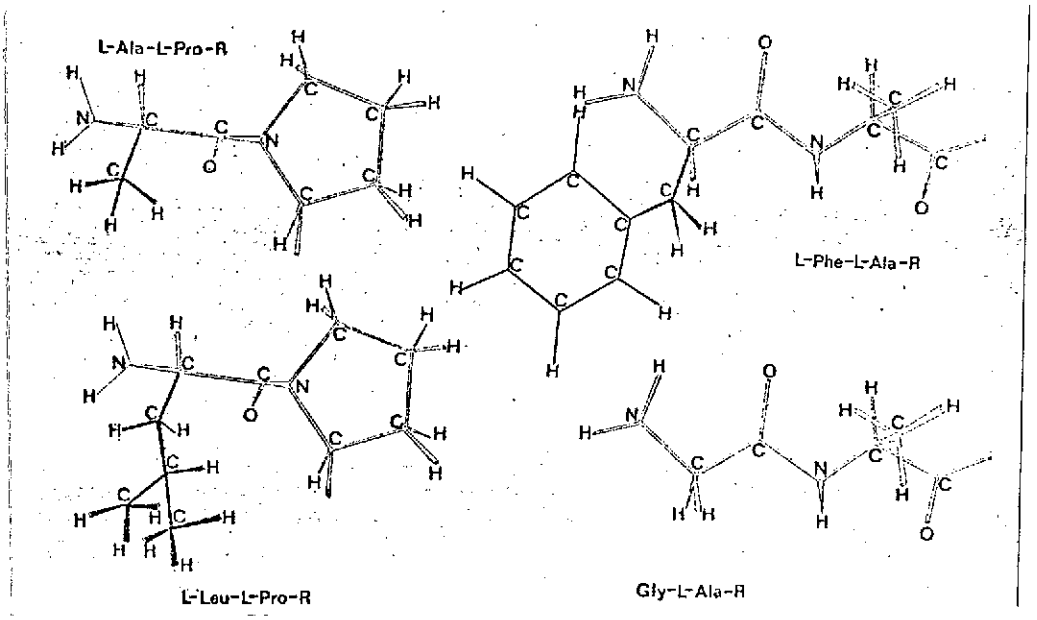

Figure 10. Structural models of the dipeptide portions of some substrates of the series L-amino acid-L-Ala-PAP-amide and L-amino acid-L-Pro-PAP-amide.

arrangement of the amino acid residues, we can see that in the substrate series L-amino acid-L-Pro-arylamide, optimal hydrolysis occurs with a relatively compact over-all structure for the substrate molecule, due to the rigid arrangement of the pyrrolidine ring. In contrast, in the substrate series L-amino acid-L-Ala-arylamide, a molecule is split best if it is characterized by a flexible arrangement of both amino acid residues. It is also of interest that representatives of both substrate types are hydrolyzed with about the same specific activity, e. g., L-Leu-L-Pro-PAP-amide and L-Phe-L-Ala-PAP-amide, even though they differ basically not only in the direct point of enzymic attack but also in their over-all spatial arrangement in the enzyme-substrate interaction.

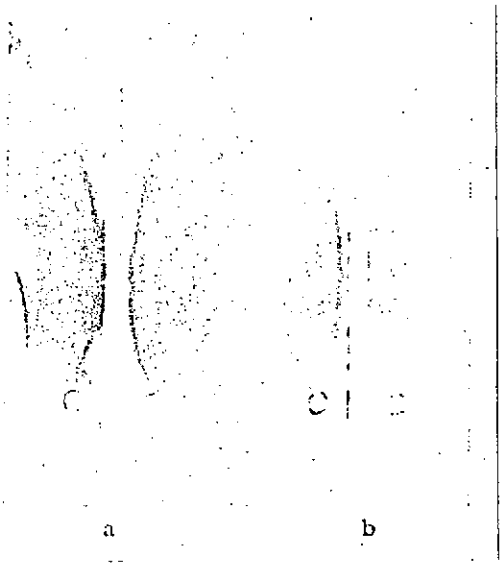

Figure 11. Immunoelectrophoretic demonstration of the dipeptidylaminopeptidase activity in the precipitation lines of the antigen-antibody reaction.

a. Gly-L-Pro-β-naphthylamide hydrolysis.
b. L-Ala-L-Ala-β-naphthylamide hydrolysis.

Agarose gel, 1%; 0.02 M Veronal buffer, pH 8.5.

These results suggest that two active centers with high selectivity exist, or are formed, in dipeptidylaminopeptidase IV isolated from hog kidney. The fact that both types of substrate / 169 are hydrolyzed in one and the same precipitation line of the immunoelectropherogram (Figure 11) also argues for this working hypothesis, as the action of two different individual proteins can be thoroughly excluded. The hypothesis is also supported by the fact that there is quantitatively different inhibition of the hydrolysis of Gly-L-Pro-PAP-amide and L-Ala-L-Ala-PAP-amide by DFP. There is almost an order of magnitude difference in / 170 the DFP concentrations which produce 50% inhibition of the enzyme with the two substrates. But there is still no indication of whether these assumed two active centers exist completely independently of each other, become active through

mutual influence, or whether there can only be a highly specific adaptation to the two substrate types within one catalytic region of the enzyme. More studies are being prepared to clear up this situation.

5. Determination of kinetic parameters

The experiments showed that the dependence of the rate of L-Alanyl-L-Alanine-PAP-amide hydrolysis on the substrate concentration is a Michaelis-Menten function. The Michaelis constant was found to be $5.7 \cdot 10^{-5}$ from the Lineweaver-Burk diagram. The Hill coefficient is $n = 1$.

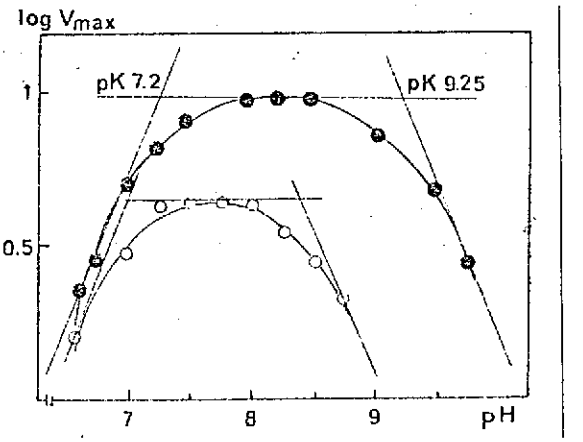

Figure 12. Plot of log V_{max} versus the pH (Dixon plot) for hydrolysis of L-Ala-L-Ala-PAP-amide (concentration $1 \cdot 10^{-4}$ M) by dipeptidylaminopeptidase at 30°C.
●——● = under pH stat conditions (no buffer);
○——○ = in presence of Theorell-Stenhagen buffer.

A nearly bell-shaped dependence of the theoretical maximum velocity on the pH is found from the Dixon plot (Figure 12).

The kinetic measurements of the reaction rate as a function of the pH show that real measurements could be obtained only under pH-stat conditions. Buffer ions such as citrate and phosphate (see above) inhibit the enzymatic activity by more than 50% even at a concentration of $2.5 \cdot 10^{-2}$ M, and lead to a shift of the dissociation constants of functional groups in the dipeptidylaminopeptidase molecule (Figure 12).

According to Koshland and Neet [35], the bell-shaped curve in the plot of log V_{max} versus pH indicates acid-base catalysis.

The experimentally determined pK values (pK_1 = 7.25 and pK_2 = 9.25) differ significantly from the pK value of the substrate (6.7) and must, therefore, be assigned to the free enzyme or to the enzyme-substrate complex. They indicate possible participation of imidazole residues (histidine) and hydroxyl groups (serine) in the catalytic process. / 171

In contrast to V_{max}, the K_m value is practically independent of the pH.

6. Step by step mechanism of substrate hydrolysis

Some publications [25-29] have already reported on the stepwise degradation of di- and oligo-peptides by aminopeptidases, proceeding from the N-terminal end. Ellis and Nuenke [30] first observed a step-by-step mechanism of substrate hydrolysis by dipeptidylaminopeptidase III. In agreement with that, it can be shown in the following studies that the microsomal dipeptidylaminopeptidase IV isolated from hog kidney is also able to degrade oligopeptidylarylamides step by step from the N-terminal end. In the case of enzymic degradation of the substrate L-Alanyl-L-Alanyl-L-Alanyl-L-Alanine-PAP-amide by

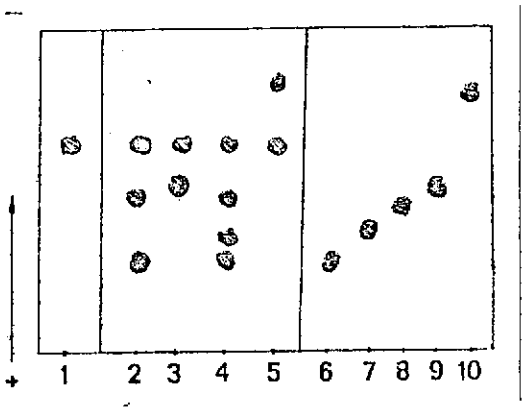

Figure 13. Separation of the hydrolysis products in the hydrolysis of L-Ala-L-Ala-PAP-amide (2), L-Ala-L-Ala-L-Ala-PAP-amide (3), L-Ala-L-Ala-L-Ala-L-Ala-PAP-amide (4), and L-Ala-L-Ala-L-Lys-NH_2 by dipeptidylaminopeptidase IV, using paper electrophoresis. Reference substances: 1 = L-Ala-L-Ala; 6 = p-aminoazobenzene; 7 = L-Ala-L-Ala-L-Ala-PAP-amide; 8 = L-Ala-L-Ala-PAP-amide; 9 = L-Ala-PAP-amide; 10 = L-Ala-L-Ala-L-Lys-NH_2.

Substrate incubation in the enzyme solution: 4 hrs; 6 hrs for L-Ala-L-Ala-L-Lys-NH_2. Electrophoresis duration: 75 minutes; 30 minutes for L-Ala-L-Ala-L-Lys-NH_2.

dipeptidylaminopeptidase IV, paper electrophoretic analysis of the hydrolysis products gives the patern shown in Figure 13.

After an incubation period of 4 hours, L-Alanyl-L-alanine-PAP-amide is clearly demonstrated, along with the substrate used and the hydrolysis products L-Alanyl-L-Alanine and p-aminoazobenzene. Similarly, the dipeptidylaminopeptidase hydrolyzes the substrate L-Alanyl-L-Alanyl-L-alanine-PAP-amide to the end products L-Alanyl-L-Alanine and L-Alanine-PAP-amide. /172
No p-aminoazobenzene appears here because L-Alanine-PAP-amide is no longer split by the dipeptidylaminopeptidase.

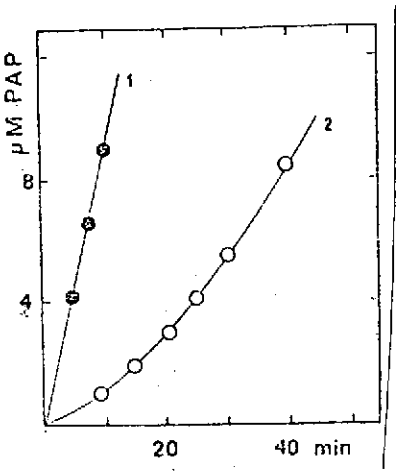

Figure 14. Plot of the increase in concentration of the hydrolysis product p-aminoazobenzene with time in the hydrolysis of L-Ala-L-Ala-PAP-amide (1) and L-Ala-L-Ala-L-Ala-L-Ala-PAP-amide (2) by dipeptidylaminopeptidase. Substrate concentration: $9 \cdot 10^{-5}$ M; Theorell-Stenhagen buffer, pH 7.7.

As Figure 14 shows, the increase in concentration of the hydrolysis produce, p-aminoazobenzene, from enzymatic splitting of L-Alanyl-L-Alanine-PAP-amide is linear in the first minutes of incubation, in spite of relatively large hydrolysis rates. In contrast, the increase in concentration of the final hydrolysis product (p-aminoazobenzene) is from the first an exponential function of the time in hydrolysis of the substrate L-Alanyl-L-Alanyl-L-Alanyl-L-Alanine-PAP-amide. These results show that the hydrolysis of oligopeptide-PAP-amides by hog kidney dipeptidylaminopeptidase IV proceeds so that N-terminal dipeptides are split off "step-by-step" in the course of the reaction [29].

7. Determination of the molecular weight and isoelectric point of dipeptidylaminopeptidase IV

In order to determine the molecular weight of the dipeptidylaminopeptidase IV (estimation), the logarithms of the molecular weights of the calibration proteins were plotted versus the elution quotients, V_e/V_0, which were determined. This gave an approximate molecular weight of 230,000 for the dipeptidylaminopeptidase.

To determine the isoelectric point, the highly purified dipeptidylaminopeptidase, rechromatographed on Sepharose 6B, was subjected to electrophoresis on agarose gel along with human serum albumin at various pH values. Graphical evaluation of the migration rates in the electrical field gave an isoelectric point of 6.4.

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